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(54) Title: HIGH LYSINE CORN

(57) Abstract

A corn plant and the seed therefrom is disclosed which carries the Zpr10/(22) gene so that wholekernel lysine and/or threonine levels are substantially elevated above the levels of lysine and/or threonine in the corresponding wild-type corn plant which does not contain said gene. Preferably, the gene is contributed by line BSSS53.

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HIGH LYSINE CORN

Background of the Invention

Lysine, an amino acid essential in the diet of humans and monogastric animals, is among the three most limiting nutrients in most of the cereal crops. Consequently, grain-based diets, such as those based on corn, barley, wheat, rice, maize, millet, sorghum and the like, must be supplemented with more expensive synthetic lysine or with lysine-containing oilseed protein meals. Unsupplemented corn feed is primarily limiting in lysine and tryptophan, while corn-legume mixtures often are deficient in methionine. Corn is deficient in methionine for some uses such as poultry rations. Therefore, the protein and amino acid content of corn has been the subject of numerous studies concerned with cereal quality. Increasing the lysine content of these grains or of any of the feed component crops would result in significant added value. To date, attempts to elevate lysine levels in plants have relied on conventional breeding methods and, more recently, mutagenesis and and cell culture technology.

Seed storage proteins have been defined as those proteins whose primary role is to store nitrogen and amino acids for later use by the developing seedling; no enzyme activity has been identified. These proteins are deposited in the developing seed. T. B. Osborne, in The Vegetable Proteins, Longmans, Green Co., London (1912), defined four classes of seed storage proteins entirely in terms of their solubility: albumins (water-soluble), globulins (salt-soluble), prolamines (alcohol-soluble) and glutelins (alkali-soluble). While the prolamine class (zein) is the most abundant in corn, it is not nutritionally balanced. Zein is strongly deficient in essential amino acids, most notably lysine and tryptophan, while it contains

large amounts of glutamic acid, leucine, proline and alanine.

Zein extracts contain a mixture of polypeptides with relative molecular weights of 27, 22, 19, 15 and 10 kD. The 22 and 19 kD proteins are extracted in alcohol alone (zein-1 extract), whereas the 27, 15 and 10 kD proteins require the presence of a reducing agent (zein-2 extract). The 22 kD and 19 kD components contain similar levels of amino acids. The 15 kD and 10 kD components contain higher levels of methionine, cysteine, tyrosine and glycine.

In addition to the many structural zein genes, loci influencing the accumulation of entire classes of zein protein have been identified. These genes have been termed "regulatory," although the exact mechanisms underlying the protein changes have not been identified. Mertz et al., in Science, 145, 279 (1964) recognized the opaque-2 endosperm mutant as the first maize variant with a modified protein composition in the kernels. The concentration of lysine in opaque-2 kernels is approximately double that found in near-isogenic counterparts. Additional endosperm mutants containing higher concentrations of nutritionally favorable albumins, globulins, and glutelins and lower concentrations of zeins have since been characterized by Misra et al., Cereal Chem., 56, 497 (1975): opaque-7, sugary-1, shrunken-1, shrunken-2, shrunken-4, floury-2 and brittle-1. In addition to increased levels of lysine, sugary-1 and floury-2 mutants contain 21-36% and 50-70% more methionine in endosperm proteins, respectively. As discussed hereinabove, increased levels of methionine are also of significant interest. However, while nutritionally superior, these mutants are associated with reduced yields, disease susceptibility and poor grain quality, limiting their agronomic usefulness.

In particular, the opaque-2 endosperm mutant has been found to yield less grain, to retain higher moisture at harvest, and to succumb to more fungal infections and storage insect infestations than do non-variant maizes. The opaque-2 grain has a dull and chalky appearance, and the floury texture of its soft kernels make the grain difficult to store and mill. See National Research Council, "Quality-Protein Maize," National Academy Press, Washington, D.C., (1988), at vii-viii. Thus, in order to be agronomically useful, endosperm mutants such as opaque-2 require additional modifier genes to be incorporated prior to their use. Such modifications may significantly increase breeding time and effort.

In higher plants (as in bacteria), lysine, threonine and methionine are synthesized in a branched pathway from aspartate (Figure 1). The first and third enzymes in this pathway, aspartokinase (EC 2.7.2.4) and homoserine dehydrogenase, (EC 1.1.1.3) respectively, are known to be regulated by endproduct feedback inhibition. Lysine or the combination of lysine and threonine are known to inhibit the activity of aspartokinase, while threonine inhibits the activity of homoserine dehydrogenase. Feedback inhibition is a regulatory mechanism by which organisms, including plants, efficiently regulate the synthesis of cellular metabolites. Regulatory mutants, resistant to feedback inhibition or enzyme repression, have been well defined in prokaryotes and in lower eukaryotes. Recently, tryptophan and methionine overproducers have been isolated from plant cell cultures. Feedback inhibition mutants result in overproduction of the pathway endproduct(s) due to altered regulatory sites which do not allow normal inhibition of the enzyme. In crop plants, these mutants might provide a mechanism to increase the total synthesis of nutritionally limiting amino acids.

Equimolar concentrations of lysine and threonine have been demonstrated to inhibit plant growth (Green and Phillips, Crop Sci., 14, 827 (1974); Green and Donovan, Crop Sci., 20, 358 (1980)). Inhibition is relieved with the addition of methionine or one of its precursors, homocysteine or homoserine, indicating that the observed growth inhibition is a result of reduced methionine synthesis and subsequent methionine starvation.

Green and Phillips, in Crop Sci., 14, 827 (1974), proposed that feedback-inhibition-resistant mutants could be selected from maize callus cultures following the addition of lysine and threonine ("LT") to the culture medium. It was hypothesized that isolated mutants from this system might be insensitive to feedback inhibition, and subsequently overproduce aspartate-derived amino acids. An alternate screening system utilizing germinating kernels rather than callus culture was also described (Phillips et al., Crop Sci., 21, 601 (1981)).

Screening of inbred lines for LT resistance by germinating kernels on media supplemented with lysine-plus-threonine revealed a highly resistant strain from the Iowa Stiff Stalk Synthetic population, designated BSSS53 (Phillips et al., Crop Sci., 21, 601 (1981)). Germinated whole kernels of BSSS53 were LT resistant, whereas dissected embryos were inhibited, indicating that the endosperm was responsible for the seedling resistance. Amino acid analysis of BSSS53 kernels, along with kernels from inbreds differing in LT response, gave rise to the hypothesis that resistance is due to the relative concentrations of methionine and lysine in the kernel, with resistance being associated with a high methionine-to-lysine ratio (M/L ratio). Whole kernels of BSSS53 contain 21% more methionine than kernels of other inbreds analyzed.

Phillips and McClure, Cereal Chem., 62, 213 (1985), observed that, while the distribution of protein among the Osborne fractions varied only slightly, the methionine concentrations of the zein-2 fractions differed significantly among lines. Analysis of zein-2 fractions by SDS-PAGE indicated an increased proportion of 10 kD polypeptides in BSSS53 as compared to other inbreds. The 10 kD fraction differed not only in relative amount but also in methionine composition, with 10 kD polypeptides from BSSS53 containing approximately 21 mol % methionine. Ten kD polypeptides from other inbreds tested contained 9 to 19 mol % methionine.

Overaccumulation of the major 10 kD protein in BSSS53 is due to a single, semi-dominant allele at the Zpr10/(22) locus. A band detected by isoelectric focusing corresponding in position to Zp22/6 (formerly Zp6), showed linkage with the major 10 kD band, placing the gene near Gal. Separate tests showed linkage of the major 10 kD band with Gal, fl2 and wx translocation 4-9g. M. S. Benner and R. L. Phillips, in Maize Genetics Cooperation Newsletter, 60, 114 (1986), have proposed the gene symbol Zpr10/(22), which will be used hereinafter. This gene is the putative regulator of the structural gene(s) which encodes the 10 kD zein polypeptide(s). See J. A. Kirihara et al., Mol. Gen. Genet., 211, 477 (1988).

The gene Zpr10/(22) was identified by screening for resistant plants from seeds germinated on LT supplemented medium. The Zpr10/(22) mutation could not have been selected in callus culture because its associated LT resistance is dependent on an interaction of the embryo and endosperm. The Zpr10/(22) mutation thus differs from previously reported LT resistant mutants, which were selected from callus cultures, in several ways. The first report by Hibberd et al., in Planta,

148, 183 (1980), identified a callus culture which possessed resistance to LT inhibition. The cells produced an altered key enzyme, aspartokinase, and had elevated lysine in the free amino acid pool. No seed could be recovered. The second report (Hibberd and Green, Proc. Natl. Acad. Sci. U.S.A., 79, 559 (1982)) described an LT resistant callus culture and resistant regenerated plants. This mutant, designated Ltr*19, conferred high threonine levels in the free amino acid pool of kernels from resistant plants, but lysine levels were not elevated. A similar mutant, designated Ltr*20, has been identified by the same procedure (Diedrick, Ph.D. Thesis, University of Minnesota (1984)). At the present time, it is difficult to obtain these Ltr*19 and Ltr*20 mutations in homozygous form, due to negative associated traits such as defective embryos, poor germination and the like.

Therefore, a need exists to develop nutritionally-improved corn lines with increased concentrations of amino acids such as lysine and/or threonine.

Brief Description of the Invention

The present invention provides a corn plant which is homozygous for the Zpr10/(22) gene, and which exhibits whole kernel amino acid levels which are elevated over the levels present in the corresponding wild-type plant which does not comprise the Zpr10/(22) gene. The corn plant of the invention is produced by transferring the Zpr10/(22) gene from the high methionine strain, BSSS53, into other genetic backgrounds comprising the 10 kD zein protein structural gene by plant breeding procedures. The presence of the gene at each step is recognized by the overproduction of a specific protein (the 10 kD methionine-rich zein protein), e.g., by using isoelectric focusing gels.

Although the Zpr10/(22) gene was hypothesized to be responsible for increased zein methionine levels in donor line BSSS53 (genotype Zpr10/(22)/Zpr10/(22), surprisingly, substantially elevated levels of lysine were also found in the whole kernels of the Zpr10/(22)/-Zpr10/(22) lines derived from BSSS53. Furthermore, when inbred lines A619, A632, B73 or Mo17 were employed as the recurrent parent to produce an F1 hybrid, which was in turn backcrossed twice into the respective inbred line, and the heterozygous BC2 individuals selfed twice to yield homozygous plants, the average levels of asparagine, glutamine, serine, histidine, glycine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine and leucine, as well as lysine and threonine, were substantially (about 15-30%) elevated over the levels determined for the corresponding wild-type (+/+) plant.

Therefore, the present invention is directed to a corn plant, and the seed therefrom, which comprises and preferably is homozygous for the Zpr10/(22) gene, so that the whole-kernel levels of amino acids lysine, threonine or both lysine and threonine are substantially elevated above the levels of said amino acids in the corresponding wild-type hybrid corn plant which does not contain said gene. The corn plant of the invention can be produced by a process comprising:

(a) crossing a member of the Iowa Stiff Stalk Synthetic population, BSSS53, comprising the Zpr10/(22) gene, onto an inbred recurrent parent line which does not comprise said Zpr10/(22) gene (e.g., A619, A632, B73 or Mo17 and the like), to yield an F1 hybrid;

(b) twice backcrossing the F1 hybrid with the recurrent parent line;

(c) identifying BC2 progeny comprising said Zpr10/(22) gene; and

(d) selfing said progeny at least twice to produce a homozygous BC2, S2 line, wherein some ears contain only Zpr10/(22)/Zpr10/(22) kernels, so that the whole kernel amino acid levels of lysine, threonine or of both lysine and threonine of said kernels are substantially elevated above the levels of said amino acids in the corresponding BC2, S2 progeny which do not contain the Zpr10/(22) gene (see Figure 2).

The presence of the gene at each step is recognized by the overproduction of the 10 kD methionine-rich zein protein. Amino acid analyses demonstrate uniformly high total lysine and threonine in derived lines containing the Zpr10/(22) gene. Selfing the progeny in step (d) at least 5-6 times yields an inbred corn plant as that term is understood by the art.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the L-lysine, L-threonine, and L-methionine biosynthetic pathway in higher plants.

Figure 2 is a diagrammatic representation of the crossing path employed to produce Zpr10/(22)/-Zpr10/(22) and +/+ corn ears for amino acid comparisons.

Figures 3a and 3b present a diagrammatic representation of the crossing path which can be employed to recover near-isogenic lines producing ears containing all Zpr10/(22)/Zpr10/(22) kernels, from the BC2 genotype shown in Figure 2.

Detailed Description of the Invention

The general goals and techniques of hybrid corn breeding are disclosed in U.S. Patent No. 4,731,499, the disclosure of which is incorporated by reference herein. Specifically, the goal of plant breeding is to combine in a single variety/hybrid various desirable traits of the parental lines. For field crops, these traits may include resistance to diseases and insects, tolerance to heat and drought, reducing the time to crop maturity, greater yield and better agronomic quality.

Field crops are bred through techniques that take advantage of the plant's method of pollination. A plant is self-pollinating if pollen from one flower is transferred to the same or another flower of the same plant. A plant is cross-pollinated if the pollen comes from a flower on a different plant.

Plants that have been self-pollinated and selected for type for many generations become homozygous at almost all gene loci and produce a uniform population of true breeding progeny. A cross between two homozygous plants from differing backgrounds or two homozygous lines produce a uniform population of hybrid plants that may be heterozygous for many gene loci. A cross of two plants that are each heterozygous at a number of gene loci will produce a population of hybrid plants that differ genetically and will not be uniform.

Corn plants (Zea mays L.) can be bred by both self-pollination and cross-pollination techniques. Corn has male flowers, located on the tassel, and

female flowers, located on the ear, on the same plant. Natural pollination occurs in corn when wind blows pollen from the tassels to the silks that protrude from the tops of the incipient ears.

The development of corn hybrids requires the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection breeding methods are used to develop inbred lines from breeding populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which new inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential.

Pedigree breeding starts with the crossing of two genotypes, each of which may have one or more desirable characteristics that is lacking in the other or which complement the other. If the two original parents do not provide all of the desired characteristics, other sources can be included in the breeding population. In the pedigree method, superior plants are selfed and selected in successive generations. In the succeeding generations, the heterozygous condition gives way to homogeneous lines as a result of self-pollination and selection. Typically, in the pedigree method of breeding, five or more generations of selfing and selection is practiced. $F_1 \rightarrow F_2$; $F_2 \rightarrow F_3$; $F_3 \rightarrow F_4$; $F_4 \rightarrow F_5$, etc.

Backcrossing can be used to improve an inbred line. Backcrossing transfers a specific desirable trait from one inbred or source to an inbred that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent)

to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question. The progeny of this cross is then mated back to the superior recurrent parent (A) followed by selection in the resultant progeny for the desired trait to be transferred from the non-recurrent parent. After five or more backcross generations with selection for the desired trait, the progeny will be heterozygous for loci controlling the characteristic being transferred, but will be like the superior parent for most or almost all other genes. The last backcross generation would be selfed to give pure breeding progeny for the gene(s) being transferred.

A hybrid corn variety is the cross of two inbred lines, each of which may have one or more desirable characteristics lacked by the other or which complement the other. The hybrid progeny of the first generation is designated F_1. In the development of hybrids, only the F_1 hybrid plants are sought. The F_1 hybrid is more vigorous than its inbred parents. This hybrid vigor, or heterosis, can be manifested in many ways, including increased vegetative growth and increased yield.

The development of a hybrid corn variety involves three steps: (1) the selection of superior plants from various germplasm pools; (2) the selfing of the superior plants for several generations to produce a series of inbred lines which, although different from each other, each breed true and are highly uniform; and (3) crossing the selected inbred lines with unrelated inbred lines to produce the hybrid progeny (F_1). During the inbreeding process, the vigor of the lines decreases. Vigor is restored when two unrelated inbred lines are crossed to produce the hybrid progeny (F_1). An important consequence of the homozygosity and homogeneity of the inbred lines is that the hybrid between

any two inbreds will always be the same. Once the inbreds that give the best hybrid have been identified, the hybrid seed can be reproduced indefinitely as long as the homogeneity of the inbred parent is maintained.

A single cross hybrid is produced when two inbred lines are crossed to produce the F_1 progeny. A double cross hybrid is produced from four inbred lines crossed in pairs (AXB and CXD) and then the two F_1 hybrids are crossed again (AXB)X(CXD). Much of the hybrid vigor exhibited by F_1 hybrids is lost in the next generation (F_2). Consequently, seed from hybrid varieties is not used for planting stock.

Hybrid corn seed can be produced by manual detasseling. Alternative strips of two inbred varieties of corn are planted in a field, and the pollen-bearing tassels are removed from one of the inbreds. Providng that there is sufficient isolation from sources of foreign corn pollen, the ears of the detasseled inbred (female) will be fertilized only by pollen from the other inbred (male), and the resulting seed is therefore hybrid and will form hybrid plants.

The laborious detasseling process can be avoided by using cytoplasmic male-sterile (CMS) inbreds. Plants of a CMS inbred are fertilized with pollen from another inbred that is not male-sterile. Pollen from the second inbred can contribute genes that make the hybrid plants male-fertile. Usually seed from detasseled normal corn and CMS produced of the same hybrid seed is blended to insure that adequate pollen loads are available for fertilization when the hybrid plants are grown.

The invention will be further described by reference to the following detailed example.

Example I - Production of Homozygous Zpr10/(22)/Zpr10/(22) Corn Plants

A. Crossing BSSS53 with selected inbred lines.

Inbred lines A619, A632, B73 and Mo17 were chosen as recurrent parents in the backcrossing of Zpr10/(22) into elite materials (Figure 2). These lines were obtained from J. L. Geadelmann, University of Minnesota, and are widely available, for example, from Illinois Foundation Seeds, Inc., Box 722, Champaign, Illinois, 61820. The above lines were crossed as female with BSSS53, a random line isolate from the Iowa Stiff Stalk Synthetic population; the present line being descended from material provided to J. L. Geadelmann, University of Minnesota by A. Hallauer, USDA Agricultural Research Service, Iowa State University, Ames, Iowa. Each F1 was subsequently backcrossed twice as female to the respective male recurrent parent. The presence of Zpr10/(22) was detected in heterozygous BC2 progeny due to its ability to cause overaccumulation of the major 10 kD zein protein, as visualized on isoelectric focusing gels (described below). Heterozygous BC2 individuals were selfed to produce segregating seed, which were again selfed. Ears from homozygous Zpr10/(22) (Zpr10/(22)/-Zpr10/(22)) and wild-type (+/+) plants were identified by their failure to produce segregating seed. Homozygous Zpr10/(22) kernels contained elevated 10 kD protein.

B. Protein Extraction and Isoelectric Focusing

Whole kernels or portions thereof were ground in a mill similar to that described by Paulis and Wall, in Cereal Chem., 56, 497 (1979). The zein-1 fraction was extracted twice from 50 mg of meal with 0.5 ml 70% ethanol for 30 min at room temperature. The zein-2

fraction was subsequently removed with one ml ethanol containing 1% 2-mercaptoethanol for 30 min at room temperature. In preparation for isoelectric focusing, 75 µl of zein-2 extract was dried in vacuo and resuspended in 10 µl 70% ethanol, 1% 2-mercaptoethanol. Proteins were loaded onto gels consisting of: 5% acrylamide, 6.4 M urea, 2% carrier ampholytes (Servalyte 5-8), and 0.3 ml 10% ammonium persulfate per 60-ml gel to initiate polymerization as disclosed by Kirihara et al., Mol. Gen. Genet., 211, 477 (1988). Isoelectric focusing was performed for 3 hr at 10°C with 25 W constant power. Proteins were visualized by precipitation with 10% trichloroacetic acid.

C. Amino Acid Analysis

Fifty kernels were removed from one each of the following ears, identified as described hereinabove: Zpr10/(22)A619, wild-type A619, Zpr10/(22)A632, wild-type A632, Zpr10/(22)B73, wild-type B73, Zpr10/(22)Mo17 and wild-type Mo17. Samples were ground to a fine powder in a Cyclone mill and defatted twice with petroleum ether (15 ml per gm meal). Thirty-five mg of defatted meal were hydrolyzed with 2 ml of 6M HCl at 110°C for 24 hr. Vials were flooded with nitrogen prior to hydrolysis to prevent oxidation. Hydrolyzed samples were dried in vacuo and amino acids redissolved in 0.2M sodium borate buffer, pH 9.5. Samples were diluted 1 part in 48 with sodium borate prior to analysis on a Waters HPLC analyzer.

D. Results

Elevation of whole-kernel amino acid levels are summarized in Table 1, below.

Table 1

Amino Acid Levels

Asparagine

Line	nM asp/mg defatted meal Wild-type	Zpr	% increase
A619	49.92	66.19	33
A632	54.94	62.02	13
B73	51.68	50.74	-2
Mo17	40.08	50.02	25
Average	49.98	57.08	14

Glutamine

Line	nM glu/mg defatted meal Wild-type	Zpr	% increase
A619	144.38	200.02	39
A632	200.03	235.52	18
B73	177.60	180.93	2
Mo17	102.15	130.76	28
Average	162.60	186.15	15

Serine

Line	nM ser/mg defatted meal Wild-type	Zpr	% increase
A619	24.03	35.12	46
A632	30.90	35.50	15
B73	27.06	29.02	7
Mo17	17.86	20.96	17
Average	25.77	30.57	19

Histidine

Line	nM his/mg defatted meal Wild-type	Zpr	% increase
A619	14.18	24.84	75
A632	23.74	30.06	27
B73	20.76	22.45	8
Mo17	10.57	15.04	42
Average	18.30	23.73	30

Glycine

	nM gly/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	29.64	47.87	62
A632	37.90	44.43	17
B73	34.00	37.25	10
Mo17	27.52	29.55	7
Average	33.00	39.99	21

Threonine

	nM thr/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	21.17	30.58	45
A632	28.01	33.57	20
B73	24.62	25.90	5
Mo17	17.32	20.25	17
Average	23.49	28.00	19

Arginine

	nM arg/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	16.54	27.22	65
A632	24.08	28.44	18
B73	19.44	20.48	5
Mo17	16.24	18.48	14
Average	19.61	23.81	21

Alanine

	nM ala/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	79.47	115.40	45
A632	98.81	113.92	15
B73	90.95	91.75	1
Mo17	58.31	72.55	24
Average	84.48	99.29	18

Tyrosine

	nM tyr/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	18.16	30.85	70
A632	23.69	28.43	20
B73	20.64	20.67	0
Mo17	14.50	17.15	18
Average	19.83	24.33	23

Methionine

	nM met/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	6.00	9.41	57
A632	11.09	13.02	17
B73	8.55	8.69	2
Mo17	3.34	5.08	52
Average	7.76	9.41	21

Valine

	nM val/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	33.77	50.56	50
A632	44.75	53.41	19
B73	39.30	40.84	4
Mo17	27.71	34.22	24
Average	37.51	45.23	21

Phenylalanine

	nM phe/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	29.00	43.78	51
A632	33.96	38.89	15
B73	32.11	34.01	6
Mo17	20.93	24.49	17
Average	29.81	35.52	19

Isoleucine

	nM ile/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	25.35	35.85	41
A632	31.99	36.15	13
B73	28.97	30.36	5
Mo17	19.78	22.97	16
Average	27.32	31.72	23

Leucine

	nM leu/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	93.10	136.76	47
A632	117.68	135.72	15
B73	110.87	111.35	0
Mo17	68.37	82.97	21
Average	100.86	118.07	17

Lysine

	nM lys/mg defatted meal		
Line	Wild-type	Zpr	% increase
A619	12.61	19.55	55
A632	15.61	18.80	20
B73	14.48	15.61	12
Mo17	12.99	14.68	13
Average	13.92	17.16	23

As demonstrated by the results shown in Table 1, the average levels of all 15 amino acids extracted from kernels of the BC2, S2 Zpr10/(22)/-Zpr10/(22) lines were substantially elevated over the amino acid levels measured in the corresponding wild-type +/+ kernels.

Example II - Recovery of High Amino Acid Zpr10/(22)/Zpr10/(22) Lines

A breeding strategy which can be used to obtain near-isogenic high amino acid lines is summarized in Figures 3a and 3b.

In accord with Figures 3a and 3b, the BC2 strain shown in Figure 2 is backcrossed twice with an inbred recurrent parent line (+/+). The BC strains are screened for Zpr10/(22) kernels by extracting zein-2 protein from small pieces of individual kernels and screening for elevated levels of the 10 kD zein protein on IEF gels. Zpr10/(22) individuals are grown and are crossed to the recurrent parent to ultimately yield strain BC6. Zpr10/(22) individuals are grown and selfed twice to yield the BC6, S2 line which is near isogenic to the recurrent parent. The ears containing all Zpr10/(22)/Zpr10/(22) are identified as described above by their consistently high accumulation of the zein 10 kD protein. Therefore, the present invention is also directed to useful inbred corn plants, hybrids derived therefrom and the seeds therefrom, which share the desirable phenotypic characteristics of the BC2, S2 Zpr10/(22)/Zpr10/(22) line shown in Figure 2.

The present invention also includes cultures from which corn plants can be regenerated, including cultures of the cells, protoplasts, tissue or calli of the present plants.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A corn plant which comprises the Zpr10/(22) gene so that the whole-kernel amino acid levels of lysine, threonine or both lysine and threonine are substantially elevated above the levels of said amino acids in the corresponding wild-type corn plant which does not contain said gene.

2. The corn plant of claim 1 wherein the Zpr10/(22) gene is contributed by a member of the Iowa Stiff Stalk Synthetic population, BSSS53.

3. The corn plant of claim 1 wherein the recurrent parent is an inbred line selected from the group consisting of A619, A632, B73 and Mo17.

4. A corn plant produced by a process comprising:

(a) crossing a member of the Iowa Stiff Stalk Synthetic population, BSSS53, comprising the Zpr10/(22) gene with an inbred recurrent parent line which does not comprise said Zpr10/(22) gene, to yield an F1 hybrid;

(b) twice backcrossing the F1 hybrid with the recurrent parent line;

(c) identifying BC2 progeny comprising said Zpr10/(22) gene; and

(d) selfing said progeny at least twice to produce a homozygous BC2, S2 line, wherein some ears contain only Zpr10/(22)/Zpr10/(22) kernels, so that the whole-kernel levels of lysine, threonine or of both lysine and threonine in

said Zpr10/(22)/Zpr10/(22) kernels are substantially elevated above the levels of said amino acids in the corresponding BC2, S2 progeny which does not contain said gene.

5. The corn plant of claim 4 wherein the inbred recurrent parent is selected from the group consisting of A619, A632, B73 and Mo17.

6. The corn plant of claim 5 wherein the inbred recurrent parent is crossed as female with BSSS53.

7. The corn plant of claim 6 wherein the F1 hybrid is backcrossed twice as female to the male recurrent parent to yield the BC2 progeny.

8. The corn seed of the corn plants of claims 1 or 4.

9. A culture derived from cells, protoplasts, tissue or calli of the corn plants of claims 1 or 4.

10. An inbred corn plant with the phenotypic characteristics of the corn plant of claims 1 or 4.

11. A hybrid corn plant derived from the corn plants of claims 1 or 4.

12. A hybrid corn plant derived from the inbred corn plant of claim 10.

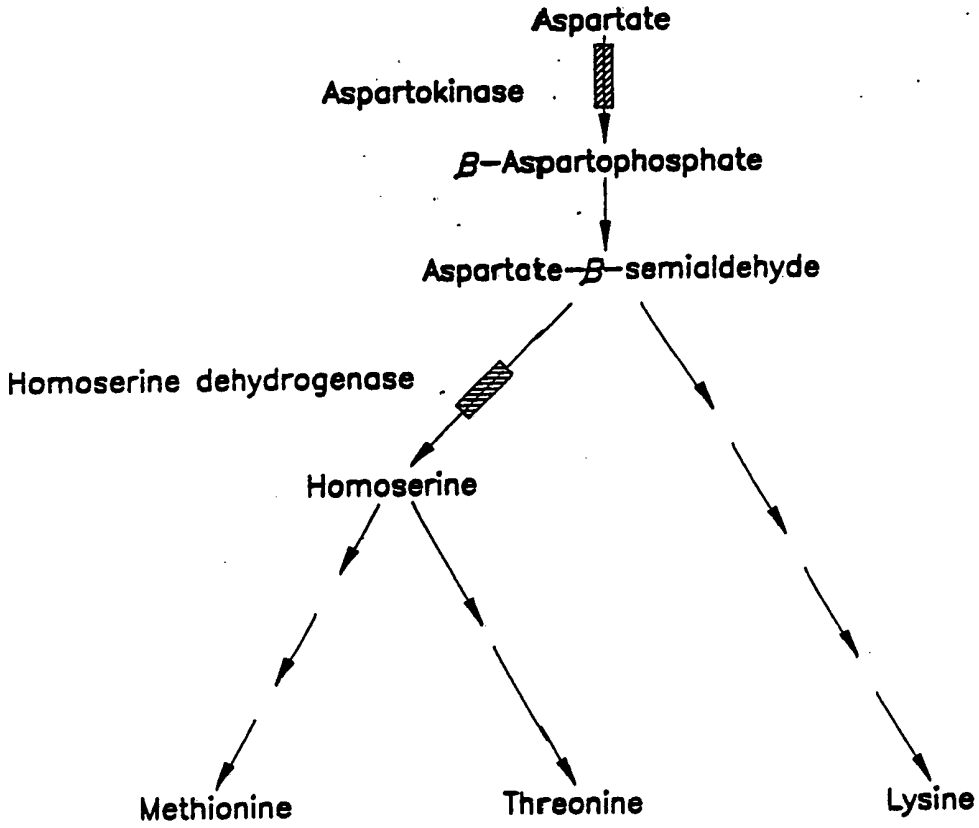
Aspartate
Aspartokinase
β-Aspartophosphate
Aspartate-β-semialdehyde
Homoserine dehydrogenase
Homoserine
Methionine
Threonine
Lysine

FIG. 1

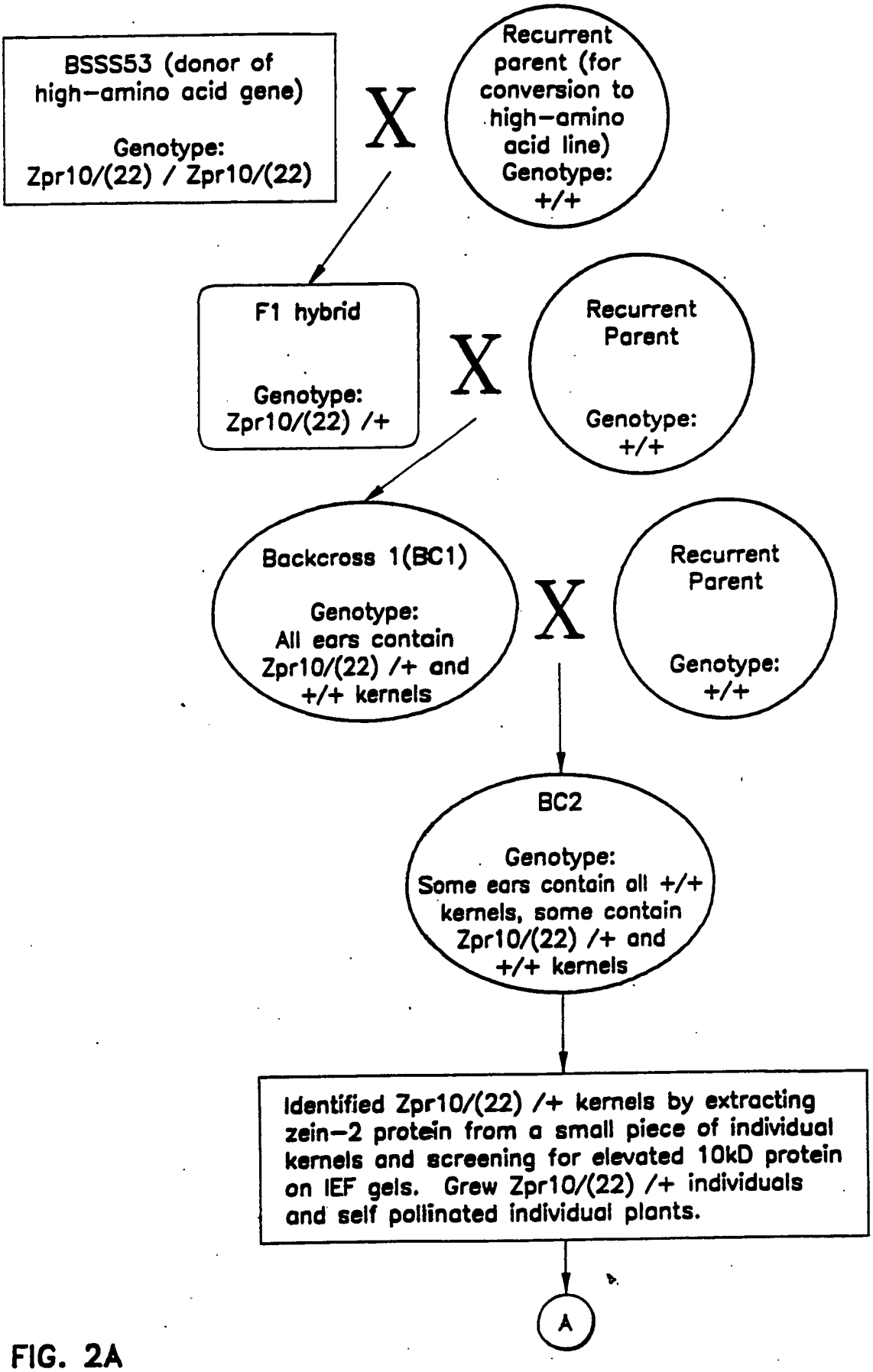
BSSS53 (donor of high-amino acid gene)
Genotype: Zpr10/(22) / Zpr10/(22)
X
Recurrent parent (for conversion to high-amino acid line) Genotype: +/+
F1 hybrid
Genotype: Zpr10/(22) /+
X
Recurrent Parent
Genotype: +/+
Backcross 1(BC1)
Genotype: All ears contain Zpr10/(22) /+ and +/+ kernels
X
Recurrent Parent
Genotype: +/+
BC2
Genotype: Some ears contain all +/+ kernels, some contain Zpr10/(22) /+ and +/+ kernels
Identified Zpr10/(22) /+ kernels by extracting zein-2 protein from a small piece of individual kernels and screening for elevated 10kD protein on IEF gels. Grew Zpr10/(22) /+ individuals and self pollinated individual plants.
A

FIG. 2A

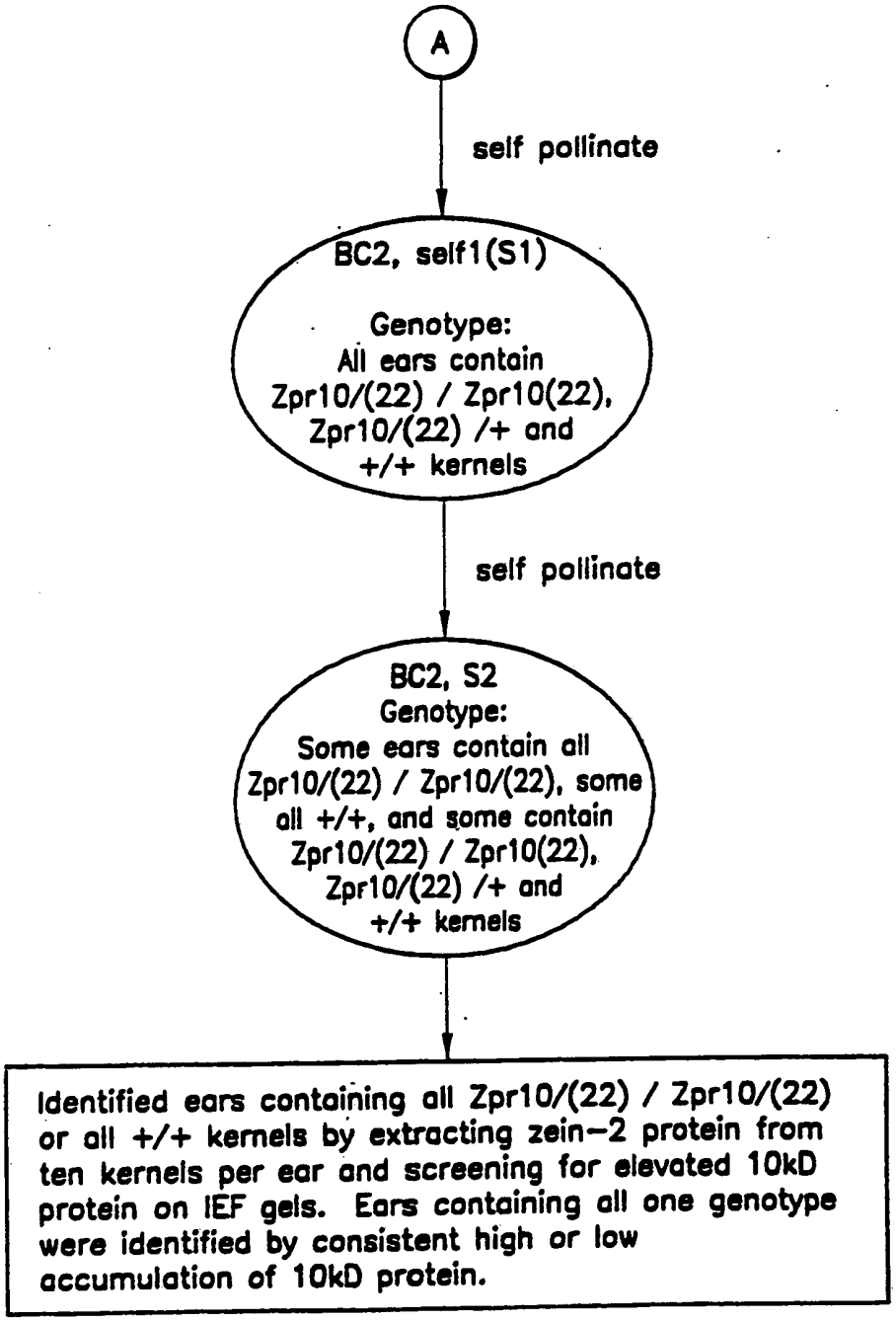
A
self pollinate
BC2, self1(S1)
Genotype:
All ears contain
Zpr10/(22) / Zpr10(22),
Zpr10/(22) /+ and
+/+ kernels
self pollinate
BC2, S2
Genotype:
Some ears contain all
Zpr10/(22) / Zpr10/(22), some
all +/+, and some contain
Zpr10/(22) / Zpr10(22),
Zpr10/(22) /+ and
+/+ kernels
Identified ears containing all Zpr10/(22) / Zpr10/(22) or all +/+ kernels by extracting zein-2 protein from ten kernels per ear and screening for elevated 10kD protein on IEF gels. Ears containing all one genotype were identified by consistent high or low accumulation of 10kD protein.

FIG. 2B

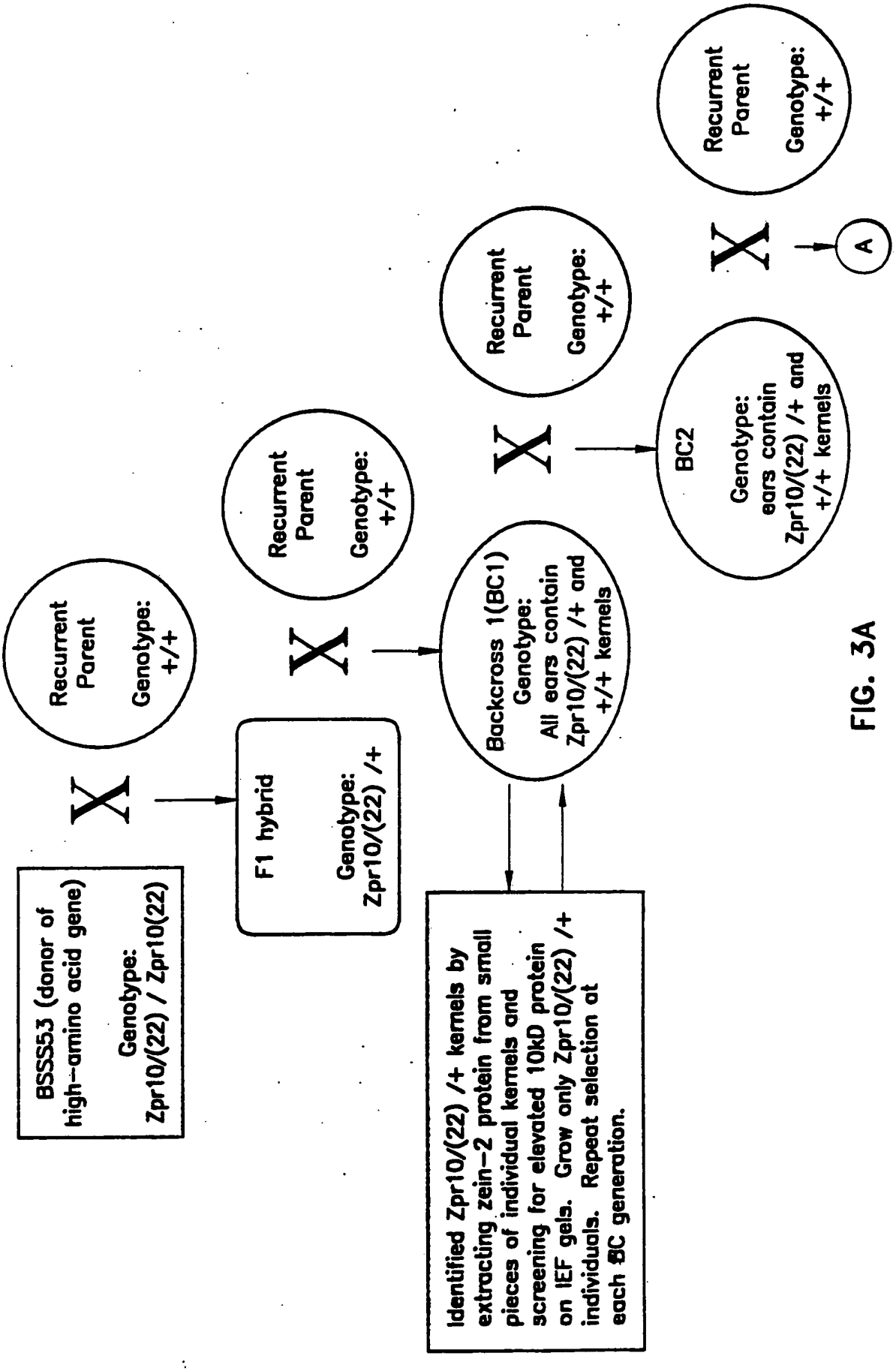
BSSS53 (donor of high-amino acid gene)
Genotype: Zpr10/(22) / Zpr10(22)
X
Recurrent Parent
Genotype: +/+
F1 hybrid
Genotype: Zpr10/(22) /+
X
Recurrent Parent
Genotype: +/+
Backcross 1(BC1)
Genotype:
All ears contain Zpr10/(22) /+ and +/+ kernels
X
Recurrent Parent
Genotype: +/+
BC2
Genotype: ears contain Zpr10/(22) /+ and +/+ kernels
X
Recurrent Parent
Genotype: +/+
A
Identified Zpr10/(22) /+ kernels by extracting zein-2 protein from small pieces of individual kernels and screening for elevated 10kD protein on IEF gels. Grow only Zpr10/(22) /+ individuals. Repeat selection at each BC generation.

FIG. 3A

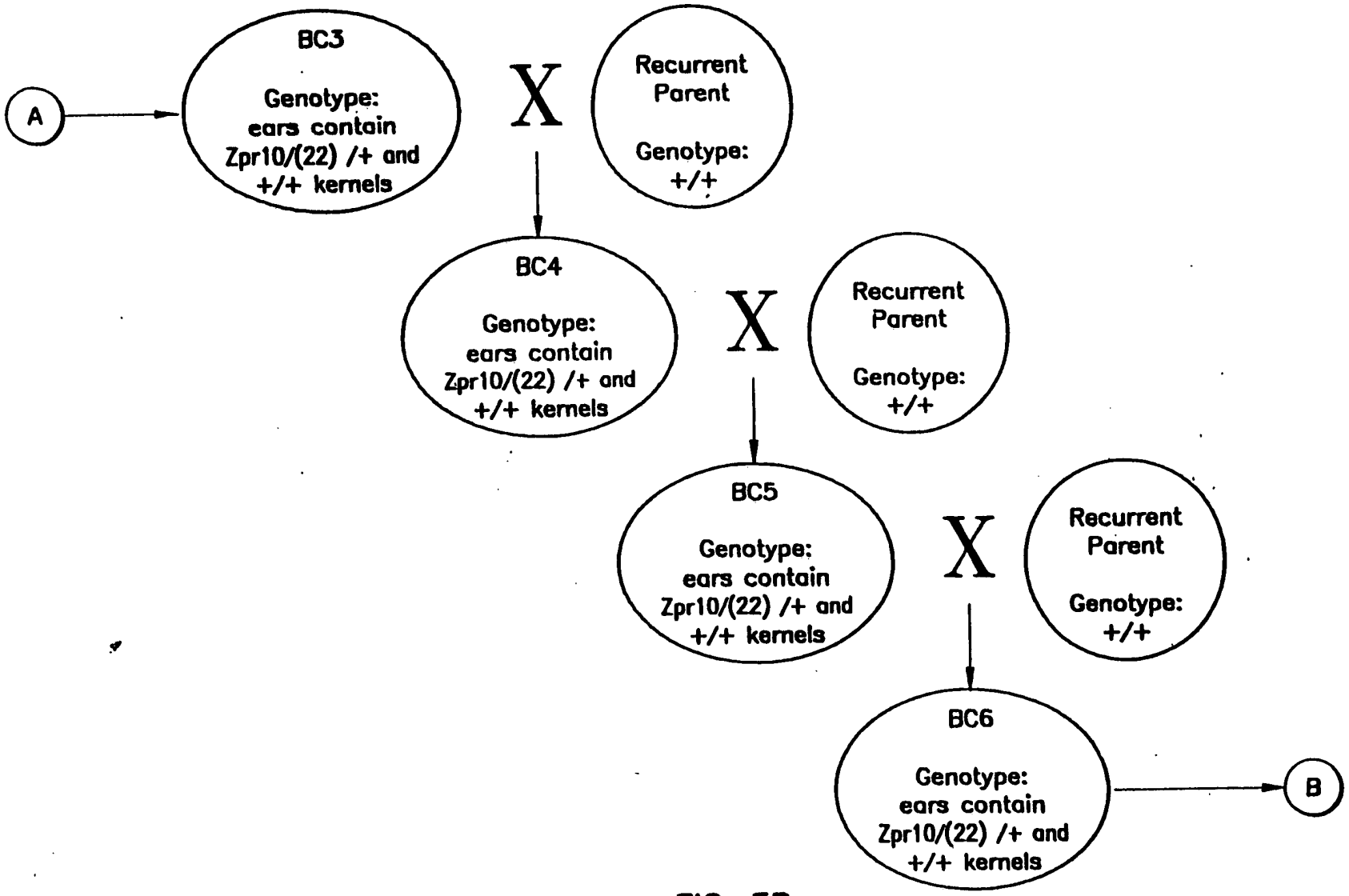
A
BC3
Genotype:
ears contain
Zpr10/(22) /+ and
+/+ kernels
X
Recurrent
Parent
Genotype:
+/+
BC4
Genotype:
ears contain
Zpr10/(22) /+ and
+/+ kernels
X
Recurrent
Parent
Genotype:
+/+
BC5
Genotype:
ears contain
Zpr10/(22) /+ and
+/+ kernels
X
Recurrent
Parent
Genotype:
+/+
BC6
Genotype:
ears contain
Zpr10/(22) /+ and
+/+ kernels
B

FIG. 3B

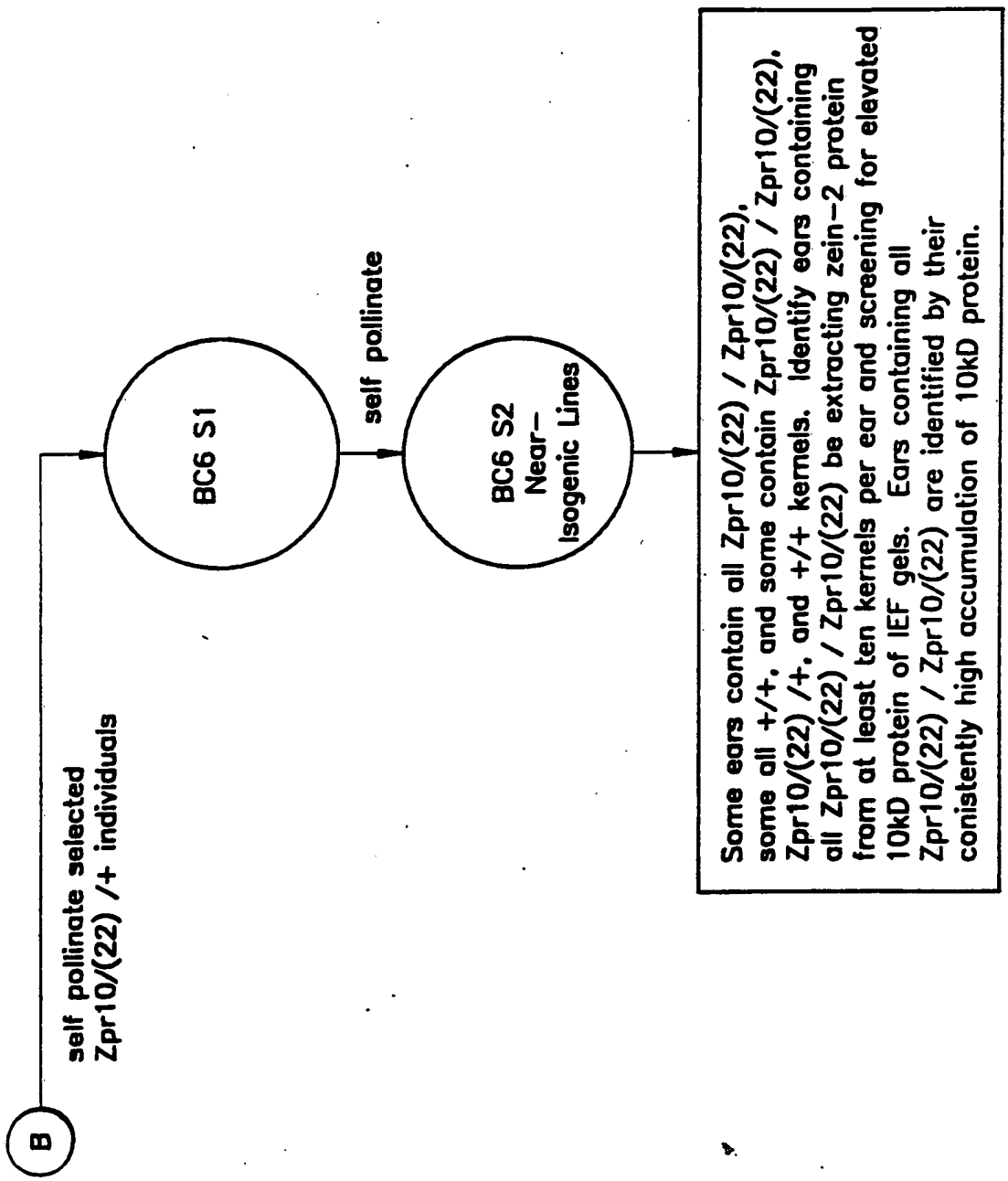
B
self pollinate selected
Zpr10/(22) /+ individuals
BC6 S1
self pollinate
BC6 S2
Near-
Isogenic Lines
Some ears contain all Zpr10/(22) / Zpr10/(22),
some all +/+, and some contain Zpr10/(22) / Zpr10/(22),
Zpr10/(22) /+, and +/+ kernels. Identify ears containing
all Zpr10/(22) / Zpr10/(22) be extracting zein-2 protein
from at least ten kernels per ear and screening for elevated
10kD protein of IEF gels. Ears containing all
Zpr10/(22) / Zpr10/(22) are identified by their
conistently high accumulation of 10kD protein.

FIG. 3C

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/03534

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A01H 1/04, 1/00; C12N 5/00
US.CL: 47/58, Dig1: 800/1; 435/240.4,240.46,240.47,240.48, 240.5

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	800/1 435/240.4 240.46.240.47,240.48, 240.5 47/58 ,Dig 1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DATA BASES: DIALOG INFORMATION SERVICES, INC., (File Agri); USPTO Automated Patent System (File USPAT, 1975-1989). See attachment for Search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Maize Genetics Cooperation Newsletter (Columbia, Missouri), Volume 60, Issued 1986, Benner, et al. "Chromosomal location of a gene controlling high methionine Zein Expression", page 114.	1-12
X Y	Cereal Chemistry (St. Paul,MN U.S.A) Volume 62, Issued 1985, Phillips, et al. "Elevated protein-bound Methionine in seeds of a maize line resistant to lysine plus threonine" pages 213-218 see the entire document but especially Tables IV-VII and IX-XI	1,2,8 1-12
A	Annual Review of Plant Physiology (Palo Alto, CA,U.S.A), volume 38, Issued 1987, Kuhlemeier, et al., "Regulation of gene expression in higher plants", see pages 234-238.	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

24 October 1989

Date of Mailing of this International Search Report

01 NOV 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

Barbara M. Chereskin

Barbara M. Chereskin

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Principles of Plant Breeding, John Wiley and Sons, Inc. (New York, U.S.A.) published 1960, see pages 265-267 and Figures 23-1 and 23-6.	4-8,10-12
Y	Cereal Tissue and Cell Cultures, Advances in Agricultural Biotechnology, Martinus Nijhoff/Dr. W. Junk Publishers (Dordrecht, The Netherlands), Published 1985, Tomes, "Cell culture, somatic embrogenesis, and plant regeneration in Maize, rice, sorghum and Millets, " pages 175-203, see the entire document.	9
Y	Crop Science (Madision, WI, U.S.A) Volume 21, Issued 1981, Phillips, et al. "Seeding screening for lysine-plus-threonine resistant maize", pages 601-607. see the entire document	1-12
A	Crop Science (Madison, WI, U.S.A), Volume 20, Issued May -June 1980 Green, et al., "Effect of aspartate-derived amino acids and aminoethyl cysteine on growth of excised mature embryos of maize," pages 358-362, see the entire document	1-12
A	Theoretical and Applied Genetics (New York, NY, U.S.A), volume 68, Issued 1984, Negrutiu, et al., "Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of Nicotiana sylvestris (spegazzini and Comes)" pages 11-20, see the entire document.	1-12
Y	Biological Abstracts, Biosciences Information Service (Philadelphia, PA, U.S.A), volume 72, Issued August 15,1981, Nesticky, et al., Lysine-Threonine inhibition as a selection System in high lysine corn (Zea Mays) breeding". Abstract number 21332	1-12

Attachment of Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

Maize, corn, inbred, hybrid, lysine, threonine, methionine, A188, A619, A632, B73, Mo17, ZPR10, B555-53, seed storage protein, overproduc?, amino acid, feedback resistan?, metabolic control, feedback (w) insensitive, feedback inhibit?, inventor's names.